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RESEARCH PAPER

Ca^{2+} signals evoked by histamine H_1 receptors are attenuated by activation of prostaglandin EP_2 and EP_4 receptors in human aortic smooth muscle cells

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BACKGROUND AND PURPOSE
Histamine and prostaglandin E_2 (PGE_2), directly and via their effects on other cells, regulate the behaviour of vascular smooth muscle (VSM), but their effects on human VSM are incompletely resolved.

EXPERIMENTAL APPROACH
The effects of PGE_2 on histamine-evoked changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and adenylyl cyclase activity were measured in populations of cultured human aortic smooth muscle cells (ASMCs). Selective ligands of histamine and EP receptors were used to identify the receptors that mediate the responses.

KEY RESULTS
Histamine, via H_1 receptors, stimulates an increase in $[Ca^{2+}]_i$ that is entirely mediated by activation of inositol 1,4,5-trisphosphate receptors. Selective stimulation of EP_2 or EP_4 receptors attenuates histamine-evoked Ca^{2+} signals, but the effects of PGE_2 on both Ca^{2+} signals and AC activity are largely mediated by EP_2 receptors.

CONCLUSIONS AND IMPLICATIONS
Two important inflammatory mediators, histamine via H_1 receptors and PGE_2 acting largely via EP_2 receptors, exert opposing effects on $[Ca^{2+}]_i$ in human ASMCs.

Abbreviations
2-APB, 2-aminoethoxydiphenyl borate; AC, adenylyl cyclase; AH6809, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid; ASMC, aortic smooth muscle cell; BW 245C, (4*S*)-(3-[(3*R*,*S*)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DDA, 2',5'-dideoxyadenosine; GW627368X, 2-[4-(4,9-diethoxy-3-oxo-1*H*-benzo[f]isoindol-2-yl)phenyl]-*N*-phenylsulfonylacetamide; HBS, HEPES-buffered saline; IP_3, inositol 1,4,5-trisphosphate; L902,688, 5-[(1*E*,3*R*)-4,4-difluoro-3-hydroxy-4-phenyl-1-buten-1-yl]-1-[6-(2*H*-tetrazol-5*R*-yl)hexyl]-2-pyrrolidinone; NAADP, nicotinic acid adenine dinucleotide phosphate; pEC_{50} (pIC_{50}), negative logarithm of the half-maximally effective (inhibitory) concentration; PLC, phospholipase C; SQ 22536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; SQ/DDA, 1 mM SQ 22536 with 200 μM DDA (used to inhibit AC); SR, sarcoplasmic reticulum; *trans*-Ned-19, (1*R*,3*S*)-1-[3-[[4-(2-fluorophenyl)piperazin-1-yl]methyl]-4-methoxyphenyl]-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylic acid; U73122, 1-[6-[[(17*β*)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U73343, 1-[6-[[(17*β*)-3-methoxyestra-1,3,5(10)-trien-17 yl]amino]hexyl]-2,5-pyrrolidinedione; VSM, vascular smooth muscle

Introduction

Histamine released from mast cells and basophils initiates inflammatory responses, notably in the vasculature where its most immediate effects are dilatation of small vessels causing increased blood flow to sites of injury. These effects are mediated primarily by H_1 histamine receptors on endothelial cells, which stimulate Gq and thereby phospholipase C (PLC) (Hill *et al.*, 1997; Jones and Kearns, 2011). In larger vessels, including human arteries, H_1 receptors directly stimulate contraction (Toda, 1987) and proliferation of vascular smooth muscle (VSM) cells (Satoh *et al.*, 1994). There are, however, differences between species in the effects of histamine on the vasculature (Wang *et al.*, 1993, and references therein).

Inflammation plays a key role in the development of atherosclerosis (Hansson, 2005; Swedenborg *et al.*, 2011). Atherosclerotic lesions in human aorta are associated with enhanced local synthesis of histamine and increased expression of H_1 receptors (Takagishi *et al.*, 1995; Tanimoto *et al.*, 2006). Furthermore, in animal models, prevention of histamine synthesis (Toda, 1987; Sasaguri *et al.*, 2005) or competitive antagonists of H_1 receptors (Miyazawa *et al.*, 1998) reduce intimal thickening after vascular injury. These observations implicate H_1 receptors of VSM in both inflammatory responses and the development of atherosclerosis and restenosis.

Prostaglandin E_2 (PGE_2), the most abundant PG in humans, is another inflammatory mediator with widespread effects that include regulation of blood vessels (Norel, 2007). Human VSM synthesizes PGE_2 (Soler *et al.*, 2000), and its synthesis is much increased in diseased human aorta (Bayston *et al.*, 2003). PGE_2 is associated with development of atherosclerosis (Gomez-Hernandez *et al.*, 2006), and EP receptors are potential targets for its treatment (Yang *et al.*, 2011). The effects of PGE_2 are predominantly mediated by four classes of GPCRs. EP_1 receptors stimulate PLC via Gq, EP_2 and EP_4 receptors stimulate adenylyl cyclase (AC) via Gs, and most splice variants of EP_3 receptors regulate Gi (Norel, 2007; Sugimoto and Narumiya 2007). There is evidence, albeit rather sparse in humans, suggesting that all four EP receptor subtypes are expressed in various VSM (Coleman *et al.*, 1994; see references in Breyer *et al.*, 2001) including EP_2 and EP_3 receptors in human aortic smooth muscle (Bayston *et al.*, 2003). Activation of EP_1, or more often EP_3, receptors causes contraction of various VSM (Jadhav *et al.*, 2004; Jones and Woodward, 2011; Kobayashi *et al.*, 2011) including human veins (Walch *et al.*, 2001) and arteries (Qian *et al.*, 1994; Jones *et al.*, 1997; references in Walch *et al.*, 2001). Stimulation of EP_2 or EP_4 receptors causes relaxation of many VSM (Jones *et al.*, 1997; Yang *et al.*, 2010), including human vessels (Qian *et al.*, 1994; Davis *et al.*, 2004; Foudi *et al.*, 2008). These responses are consistent with the common observation that PLC-coupled receptors generally cause Ca^{2+}-mediated contraction of smooth muscle, including VSM, while those that stimulate AC cause relaxation (references in Roscioni *et al.*, 2010). The latter is widely thought to be mediated by cyclic AMP-dependent protein kinase, which via phosphorylation, can attenuate Ca^{2+} signalling and/or reduce the sensitivity of the contractile apparatus to Ca^{2+} (references in Roscioni *et al.*, 2010). Collectively, these observations suggest that PGE_2 and EP receptors play important roles in the normal physiology of blood vessels, and they are implicated in various pathological states including atherosclerosis (Yang *et al.*, 2011).

The possibility that H_1 and/or EP receptors might be targeted for treatment of vascular diseases is attractive because H_1 receptor antagonists (Hill *et al.*, 1997) and PG analogues (Abramovitz *et al.*, 2000; Norel, 2007) are already established in clinical practice for other indications. Our choice of cultured VSM to investigate interactions between histamine and PGE_2 is vindicated by the experimental opportunities that would not be accessible in studies of tissues, and by evidence that development of atherosclerosis is associated with a phenotypic change of VSM from a contractile to a proliferating ('synthetic') phenotype (Orr *et al.*, 2010) similar to that of VSM in culture (House *et al.*, 2008).

In the present study, we demonstrate that in human aortic smooth muscle cells (ASMCs), activation of H_1 receptors by histamine stimulates an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) entirely via activation of inositol 1,4,5-trisphosphate (IP_3) receptors. Activation of EP_2 or EP_4 receptors substantially attenuates the Ca^{2+} signals evoked by histamine, but EP_2 receptors are largely responsible for the inhibition of Ca^{2+} signals and stimulation of AC activity by PGE_2.

Methods

Culture of human ASMCs

Human ASMCs were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) or provided by Dr Trevor Littlewood (Division of Cardiovascular Medicine, University of Cambridge) (Boyle *et al.*, 2002). Ethical committee approval for the latter was obtained from the Addenbrooke's NHS Trust. All cells were isolated from patients who died of causes unrelated to the cardiovascular system. Further details of the cells and the specific experiments for which they were used are provided in Supporting Information Table S1. Cells were cultured in DMEM supplemented with GlutaMAX-I, heat-inactivated FBS (10%), penicillin (100 units·mL^{-1}) and streptomycin (0.1 mg·mL^{-1}) at 37°C in humidified air containing 5% CO_2. Cells from ATCC were first cultured according to the supplier's instructions (in ATCC vascular cell basal medium supplemented with ATCC VSM growth kit), and then as described earlier before use in experiments. Cells were used between passages 2 and 6, during which they retained an elongated shape and immunostained for α-smooth muscle actin (not shown).

Measurement of $[Ca^{2+}]_i$ in cell populations

Confluent cultures of ASMC grown in 96-well plates were loaded in HEPES-buffered saline (HBS) with fluo-4 by incubation (1 h, 20°C) with fluo-4-acetoxymethyl ester (4 μM, Invitrogen, Paisley, UK), probenecid (2.5 mM) and pluronic F127 (0.02%, v/v) (Govindan *et al.*, 2010). After a further incubation (30 min) in HBS supplemented with only probenecid, the medium was replaced with HBS and the cells were used for experiments. HBS had the following composition (mM): NaCl 135, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 11.5 and HEPES 11.6 (pH 7.3). Fluorescence (excitation at 485 nm, emission at 525 nm) was recorded during

appropriate additions using a FlexStation 3 fluorescence spectrometer (MDS Analytical Technologies, Wokingham, UK). Fluorescence signals (F) were calibrated to $[Ca^{2+}]_i$ from $[Ca^{2+}]_i = K_D(F - F_{min})/(F_{max} - F)$, where K_D is the equilibrium dissociation constant of fluo-4 for Ca^{2+} (345 nM) (Gee *et al.*, 2000), F_{min} is the fluorescence of Ca^{2+}-free indicator [recorded from cells treated with 0.05% Triton X-100 and 10 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA) in Ca^{2+}-free HBS] and F_{max} is the fluorescence of Ca^{2+}-saturated indicator (cells treated with 0.05% Triton X-100 and 10 mM $CaCl_2$ in HBS). Ca^{2+} signals were recorded after addition of histamine alone or in the presence of additional drugs added 5 min before histamine. Further details are provided in the figure legends.

Measurement of AC activity

Two assays were used to determine AC activity in confluent cultures of ASMC. A HitHunter cAMP HS+ (DiscoveRx, Birmingham, UK) enzyme complementation assay was used for most analyses. Briefly, confluent cultures of ASMC in 96-well plates were washed twice with HBS and incubated with appropriate stimuli (5 min). Reactions were stopped by aspirating the medium followed by addition of ice-cold ethanol (100 µL). After 10 min, the ethanol was evaporated (60°C, 30 min). This heating step also inactivates an endogenous β-galactosidase-like activity that otherwise contributes to the final luminescence measurement. The cAMP contents of the extracts were then determined according to the manufacturer's instructions. Because the AC inhibitors used [1 mM SQ 22536 with 200 µM DDA (SQ/DDA)] interfere with the HitHunter assay, analysis of their effects used a ^{3}H-adenine-labelling assay in which cultures of ASMC in 24-well plates were incubated with [2,8-^{3}H] adenine (1 µCi·mL^{-1}, 1 mL per well) in DMEM for 2 h at 37°C in humidified air containing 5% CO_2. After washing twice with HBS, cells in HBS at 20°C were used for experiments. Incubations were terminated by aspirating the medium and then adding ice-cold trichloroacetic acid (5% v/v, 1 mL). After 30 min at 4°C, ^{3}H-labelled adenine nucleotides were separated using sequential Dowex 50WX4-400 (Bio-Rad, Hemel Hampstead, UK) and alumina columns (Sigma-Aldrich, Poole, UK) (Salomon *et al.*, 1974).

Analyses of EP receptor expression by quantitative PCR (QPCR)

Confluent cultures of human ASMC in 24-well plates were lysed with Fastlane cDNA kit (500 µL per well, Qiagen, Crawley, UK). QPCR reactions were conducted according to the manufacturer's instructions using Rotorgene SYBR green PCR kit (Qiagen) with an initial denaturation (95°C, 5 min) followed by 40 cycles of amplification (95°C for 5 s and 60°C for 10 s). Fluorescence was measured at the end of each cycle (Govindan *et al.*, 2010). Primers specific for EP_2 (Qiagen Quantitect Primer Assay, code: Hs_PTGER2_1_SG; Qiagen) or EP_4 receptors (Hs_PTGER4_2_SG) or, for calibration, primers for GAPDH (forward, ACCACAGTCCATGCCATCAC; reverse, TCCACCACCCTGTTGCTGTA) were used. The authenticity of each PCR product was confirmed by melting-curve analysis. Amplification efficiency (E) was calculated as 10^m, where m is the average increase in fluorescence for four cycles after the cycle threshold (C_T). Expression levels relative to the housekeeping product (GAPDH) were calculated from

$$\text{relative expression} = E^{-C_T^{EP}} / E^{-C_T^{GAPDH}}$$

Reactions were performed in duplicate with extracts from at least three different wells for each patient.

Statistical analysis

Concentration–effect relationships were individually fitted by non-linear curve fitting to Hill equations (GraphPad Prism version 5; GraphPad Software, La Jolla, CA, USA). Antagonist affinities (pK_D) were calculated from dose ratios. Two-tailed paired Student's t-test or one-way ANOVA with Bonferroni's *post hoc* test was used as appropriate, with $P < 0.05$ considered significant.

Materials

Cell culture materials, except FBS (Sigma), were from Invitrogen. Ionomycin, U73122 (1-[6-[[(17*β*)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), U73343 (1-[6-[[(17*β*)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione), SQ 22536 (9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine) and DDA (2',5'-dideoxyadenosine) were from Merck Chemicals Ltd. (Nottingham, UK). *trans*-Ned-19 [(1*R*,3*S*)-1-[3-[[4-(2-fluorophenyl)piperazin-1-yl]methyl]-4-methoxyphenyl]-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylic acid] was from Enzo Life Sciences (Exeter, UK). Thapsigargin was from Alomone Labs Ltd. (Jerusalem, Israel). Histamine dihydrochloride, cimetidine hydrochloride, mepyramine maleate, AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid), BW 245C [(4*S*)-(3-[(3*R*,*S*)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid], 2-APB (2-aminoethoxydiphenyl borate) and PGE_2 were from Sigma. Butaprost (free acid), GW627368X (2-[4-(4,9-diethoxy-3-oxo-1*H*-benzo[f]isoindol-2-yl)phenyl]-*N*-phenylsulfonylacetamide), latanoprost (free acid) and L902,688 (5-[(1*E*,3*R*)-4,4-difluoro-3-hydroxy-4-phenyl-1-buten-1-yl]-1-[6-(2*H*-tetrazol-5*R*-yl) hexyl]-2-pyrrolidinone) were from Cayman Chemicals (Ann Arbor, MI, USA). Indomethacin and sulprostone were from R&D Systems (Minneapolis, MN, USA). Ryanodine was from AbCam (Cambridge, UK). [2,8-^{3}H] adenine (36 Ci·mmol^{-1}) was from PerkinElmer (Seer Green, Bucks, UK). All other reagents were from Sigma or sources specified in the relevant Methods section. Where dimethyl sulphoxide was used as a solvent (usually 0.01% v/v; 0.1% and 0.5% v/v in Figure 1F,E respectively), it was also included in controls. Key properties of the drugs used are provided in Supporting Information Table S2. The nomenclature of receptors and ligands follows Alexander *et al.* (2011).

Results

Histamine H_1 receptors evoke Ca^{2+} signals via IP_3 receptors in human ASMCs

Histamine evoked a concentration-dependent increase in $[Ca^{2+}]_i$ in populations of human ASMC. Removal of extracellular Ca^{2+} had no effect on the initial peak response to histamine, but it abolished the very small sustained increase in $[Ca^{2+}]_i$ (Figure 1A,B). The two phases of the Ca^{2+} signal, Ca^{2+} release from intracellular stores (pEC_{50} = 6.56 ± 0.09, n = 4)

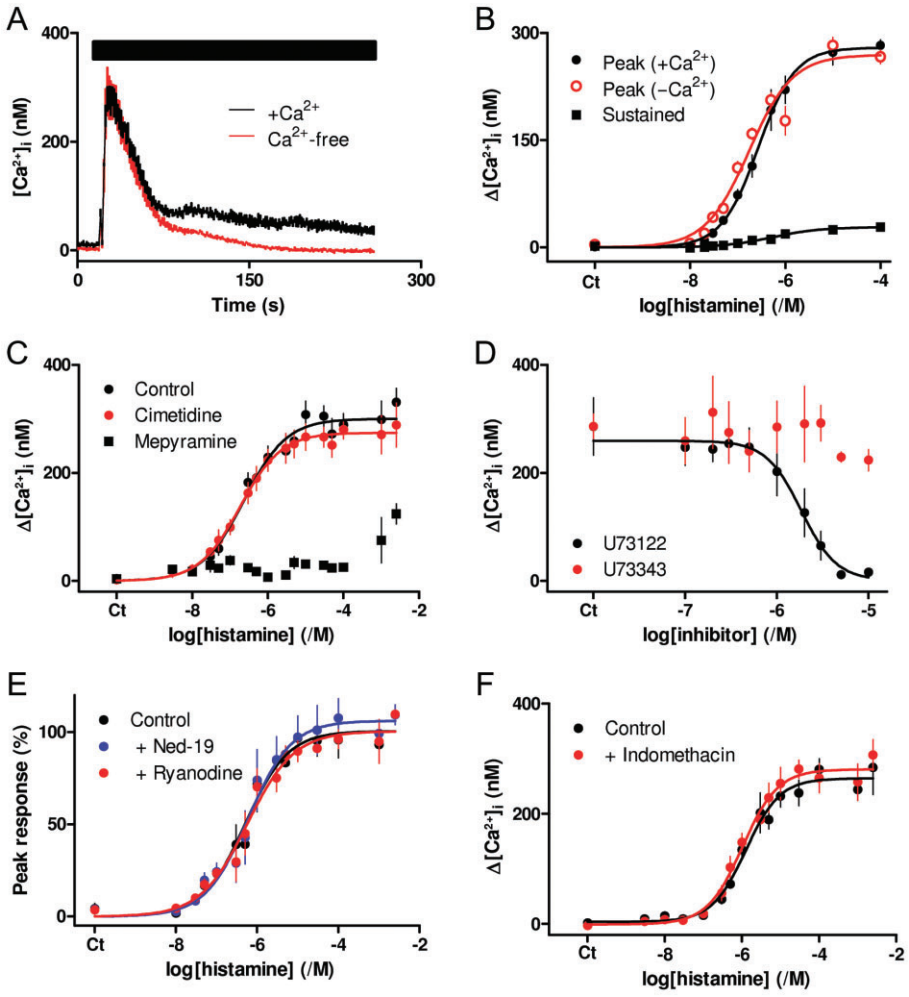

Figure 1

Histamine H_1 receptors stimulate an increase in $[Ca^{2+}]_i$ via IP_3. (A) Histamine (100 μM, bar) stimulates an increase in $[Ca^{2+}]_i$ in populations of ASMC incubated in HBS or Ca^{2+}-free HBS. Results show means ± SEM from three wells on a single 96-well plate and are typical of results from four independent plates. (B) Concentration-dependent effects of histamine on the peak $[Ca^{2+}]_i$ in the presence or absence of extracellular Ca^{2+}, and on the sustained Ca^{2+} entry (measured ~230 s after histamine addition). Results are means ± SEM from four independent plates, with one to three wells on each plate. (C) Effect of mepyramine (0.5 μM) and cimetidine (50 μM), each added 5 min before histamine, on histamine-evoked Ca^{2+} signals. Results are means ± SEM from nine independent plates, with one to three wells on each plate. (D) Effects of the indicated concentrations of U73122 and U73343 (added 5 min before histamine) on the peak increase in $[Ca^{2+}]_i$ evoked by histamine (100 μM). Results are means ± SEM from three independent plates, with two wells on each plate. (E) Effects of *trans*-Ned-19 (1 μM) and ryanodine (100 μM), each added 5 min before histamine, on the peak increase in $[Ca^{2+}]_i$ evoked by histamine. Results (percentage of the maximal response) are means ± SEM from three independent plates with one to three wells on each plate. (F) Effect of indomethacin (10 μM, added 5 min before histamine) on the peak Ca^{2+} signals evoked by histamine. Results are means ± SEM from four independent plates, each with three wells. (B–F) Ct denotes control.

and Ca^{2+} entry (pEC_{50} = 6.12 ± 0.30, n = 4), were similarly sensitive to histamine. Responses to histamine were unaffected by cimetidine (50 μM, 5 min), a selective antagonist of H_2 histamine receptors, but mepyramine (0.5 μM, 5 min), a competitive antagonist of H_1 histamine receptors (Alexander *et al.*, 2011), shifted the concentration–effect relationship to ~26 000-fold higher concentrations of histamine, suggesting a pK_D for mepyramine of ~10.7 (Figure 1C). Pre-equilibration with mepyramine, which dissociates slowly from H_1 histamine receptors, and the need to measure acute responses to histamine probably exaggerate the effect the antagonist would have at equilibrium, but the results are consistent with histamine, evoking Ca^{2+} signals via a receptor with high affinity for mepyramine and undetectable affinity for cimetidine. U73122, an inhibitor of PLC (Bleasdale *et al.*, 1990), caused a concentration-dependent inhibition (pIC_{50} = 5.76 ± 0.09, n = 3) of the Ca^{2+} signals evoked by histamine, while its inactive analogue, U73343 (≤10 μM), had no effect (Figure 1D).

Similar results, although with slightly different sensitivities to histamine and peak Ca^{2+} signals, were obtained with ASMC isolated from different patients (Supporting Information Figure S1). These differences, presumably arising from different levels of expression of H_1 receptors or downstream

signalling proteins, highlight the need for paired comparisons of experimental manipulations in subsequent analyses of human ASMC. It also justifies our presentation of some results as percentages of matched control responses.

There are no membrane-permeant selective antagonists of IP_3 receptors, although 2-APB has often been used (Taylor and Tovey, 2010). 2-APB caused a concentration-dependent inhibition of the Ca^{2+} signals evoked by either maximally or submaximally effective concentrations of histamine (Supporting Information Figure S2A–D). However, in keeping with published results (Peppiatt *et al.*, 2003), similar concentrations of 2-APB also caused a substantial loss of Ca^{2+} from the intracellular stores (Supporting Information Figure S2E,F). While the slightly greater sensitivity of the histamine responses to 2-APB is consistent with the expected involvement of IP_3 receptors, the results highlight the limitations of 2-APB as a useful antagonist of IP_3 receptors.

Ryanodine receptors and the intracellular Ca^{2+} channels that are activated by nicotinic acid adenine dinucleotide phosphate (NAADP), probably two-pore channels (Calcraft *et al.*, 2009), can also mediate Ca^{2+} release from intracellular stores, including those of VSM (Boittin *et al.*, 2002; Tugba Durlu-Kandilci *et al.*, 2010). However, caffeine (10 mM), which activates ryanodine receptors, had no effect on $[Ca^{2+}]_i$ (Supporting Information Figure S3A), and the responses to histamine were unaffected by concentrations of either *trans*-Ned-19 (1 μM, 5 min) that block responses to NAADP (Naylor *et al.*, 2009; Brailoiu *et al.*, 2010) or of ryanodine (100 μM, 5 min) that block ryanodine receptors (Zheng *et al.*, 2005) (Figure 1E).

These results demonstrate that activation of H_1 receptors by histamine stimulates Ca^{2+} release via IP_3 receptors in human ASMC, and that neither ryanodine receptors nor two-pore channels contribute to the response. Our results are consistent with widespread expression of H_1 histamine receptors in most smooth muscles (Hill *et al.*, 1997) and with evidence that histamine, via H_1 receptors, stimulates PLC and an increase in $[Ca^{2+}]_i$ in human ASMC (Satoh *et al.*, 1994). In light of subsequent evidence demonstrating effects of PGE_2 and cAMP on Ca^{2+} signals, we considered the possibility that histamine might influence Ca^{2+} signals via activation of endogenous H_2 receptors or stimulate production of endogenous prostanoids. However, neither cimetidine, a selective antagonist of H_2 receptors (Figure 1C), nor indomethacin, an inhibitor of cyclooxygenases (Figure 1F), had any effect on histamine-evoked Ca^{2+} signals in human ASMC.

PGE_2 inhibits histamine-evoked Ca^{2+} signals

PGE_2 alone had no effect on $[Ca^{2+}]_i$ (Supporting Information Figure S3B), but the Ca^{2+} signals evoked by histamine (100 μM) were attenuated by PGE_2 (10 μM, 5 min) (Figure 2A). PGE_2 decreased the sensitivity of ASMC to histamine (pEC_{50} = 6.32 ± 0.10 and 5.74 ± 0.12, n = 7, in control and PGE_2-treated cells respectively) and it reduced the maximal response by 51 ± 2% (Figure 2B). PGE_2 had similar effects on both phases of the Ca^{2+} signal: Ca^{2+} release and the small Ca^{2+} entry (Figure 2C). The effects of PGE_2 on the peak increase in $[Ca^{2+}]_i$ evoked by histamine (3 μM) were concentration dependent (pIC_{50} = 8.99 ± 0.10, n = 15; Figure 2D). Similar results, although again with some variation in absolute sensitivities, were obtained from different patients (Supporting Information Figure S1 and Table S3). PGE_2 (10 μM) did not affect the Ca^{2+} content of the intracellular stores whether assessed by addition, in Ca^{2+}-free HBS, of thapsigargin or cyclopiazonic acid to inhibit the Ca^{2+} pump (SERCA) of the sarcoplasmic reticulum (SR), or of ionomycin to release Ca^{2+} directly (Figure 2E). These results demonstrate that PGE_2 causes a concentration-dependent inhibition of the Ca^{2+} signals evoked by activation of H_1 histamine receptors without affecting the Ca^{2+} content of the SR.

Activation of EP_2 or EP_4 receptors inhibits histamine-evoked Ca^{2+} signals

PGE_2 activates many prostanoid receptors, with EP_{1-4}, DP_1 and FP receptors being the most sensitive (Abramovitz *et al.*, 2000; Alexander *et al.*, 2011). EP_1 receptors cannot mediate inhibition of histamine-evoked Ca^{2+} signals because their coupling to Gq would be expected to increase $[Ca^{2+}]_i$. Sulprostone binds selectively to EP_3 receptors, with an affinity similar to that of PGE_2. BW 245C and latanoprost are selective high-affinity agonists of DP_1 and FP receptors respectively (Abramovitz *et al.*, 2000) (Supporting Information Table S2). Sulprostone (1 μM), BW 245C (10 nM) and latanoprost (100 nM) had no effect on histamine-evoked Ca^{2+} signals (Supporting Information Figure S3C), suggesting that neither EP_3, DP_1 nor FP receptors contribute to the inhibition of Ca^{2+} signals by PGE_2. Subsequent experiments therefore assess the contributions of EP_2 and EP_4 receptors, both of which are expressed in human VSM (Qian *et al.*, 1994; Bayston *et al.*, 2003; Davis *et al.*, 2004; Foudi *et al.*, 2008).

QPCR analysis of ASMC from two patients established that transcripts for EP_2 and EP_4 receptors were expressed at similar levels. In patients a and d (Supporting Information Table S1), 48 ± 2% and 59 ± 1% of the transcripts were for the EP_2 receptor. Although we have not directly assessed protein expression, the results are consistent with comparable levels of expression of EP_2 and EP_4 receptors in human ASMC.

Butaprost, a selective agonist of EP_2 receptors (Sugimoto and Narumiya, 2007; Alexander *et al.*, 2011), caused a concentration-dependent inhibition of the Ca^{2+} signals evoked by 3 μM histamine (pIC_{50} = 8.22 ± 0.05, n = 3) (Figure 3A, Table 1). AH6809 is a poorly selective low-affinity antagonist of EP_2 receptors (pK_D = 5.9), but it does not interact with EP_4 receptors (Supporting Information Table S4). The response to butaprost was competitively antagonized by AH6809 (30 μM, ΔpIC_{50} = 1.12 ± 0.15, where ΔpIC_{50} = $pIC_{50}^{control} - pIC_{50}^{+antagonist}$), but insensitive to GW627368X (1 μM, ΔpIC_{50} = –0.21 ± 0.14), an antagonist of EP_4 receptors (Wilson *et al.*, 2006) (Figure 3A, Table 1). These results show that butaprost inhibits histamine-evoked Ca^{2+} signals via a receptor with an affinity (pK_D) for AH6809 of 5.6. This confirms that the inhibition is mediated by EP_2 receptors (Supporting Information Table S4).

L902,688 is a selective agonist of EP_4 receptors with slightly greater affinity than PGE_2 (Young *et al.*, 2004) (Supporting Information Table S4). L902,688 also caused a concentration-dependent inhibition of histamine-evoked Ca^{2+} signals (pIC_{50} = 9.52 ± 0.25, n = 3) (Figure 3B, Table 1). This inhibition was insensitive to AH6809 (30 μM, ΔpIC_{50} = –0.01 ± 0.27), but competitively inhibited by GW627368X (1 μM, ΔpIC_{50} = 2.20 ± 0.28). The latter suggests that L902,688 acts via a receptor with an affinity (pK_D) for GW627368X of

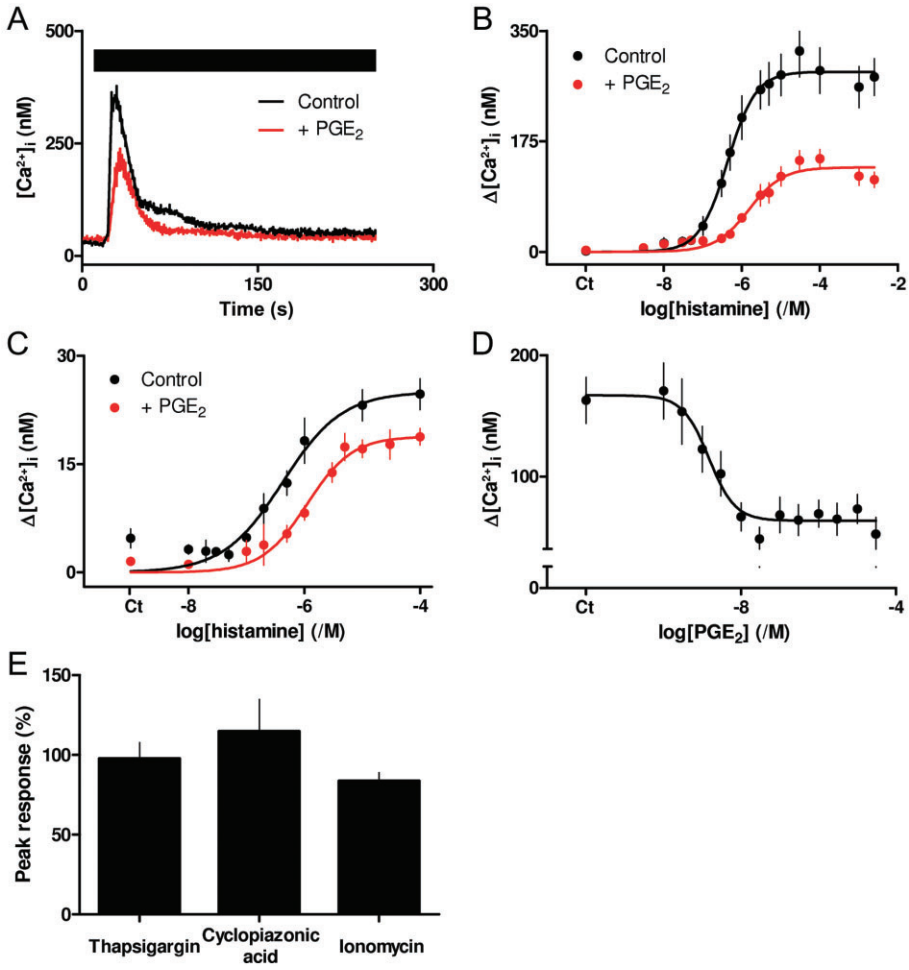

Figure 2

PGE$_2$ inhibits histamine-evoked Ca^{2+} release. (A) Ca^{2+} signals evoked by histamine (100 μM, bar) alone or with PGE$_2$ (10 μM, added 5 min before and then with histamine). Results, means ± SEM from three wells on a single plate, are typical of results from four independent plates. (B) Effect of PGE$_2$ (10 μM) on the peak Ca^{2+} signals evoked by the indicated concentrations of histamine. Results are means ± SEM from seven independent plates, each with one to three wells. (C) Effect of PGE$_2$ on the sustained Ca^{2+} signals evoked by histamine. Results are means ± SEM from 11 independent plates, each with one to three wells. (D) Effect of the indicated concentrations of PGE$_2$ (added 5 min before histamine) on the peak increase in [Ca^{2+}]$_i$ evoked by histamine (3 μM). Results are means ± SEM from 15 independent plates, with one to three wells analysed from each. (B–D) Ct denotes control. Similar results from ASMC isolated from different patients are shown in Supporting Information Figure S1. (E) Effects of pretreatment with PGE$_2$ (10 μM, 5 min) on the peak Ca^{2+} signals evoked by subsequent addition of thapsigargin (1 μM), cyclopiazonic acid (10 μM) or ionomycin (1 μM) to ASMC in Ca^{2+}-free HBS. Results (as percentages of the responses obtained without PGE$_2$) are means ± SEM from three independent plates, with seven wells analysed on each.

8.2, consistent with it being an EP$_4$ receptor (Supporting Information Table S4). We conclude that selective activation of either EP$_2$ or EP$_4$ receptors in human ASMC inhibits histamine-evoked Ca^{2+} release. For butaprost, the sensitivity of the functional response (pIC$_{50}$ = 8.22) and the published binding affinity (pK$_D$ = 7.04) differ by more than 10-fold, whereas they are more similar for L902,688 (9.5 and 9.4 respectively). This suggests that for inhibition of histamine-evoked Ca^{2+} signals, there is a greater receptor reserve for EP$_2$, than for EP$_4$, receptors.

PGE$_2$ inhibits histamine-evoked Ca^{2+} signals largely via EP$_2$ receptors

Maximally effective concentrations of PGE$_2$, butaprost or L902,688, caused similar inhibition of histamine-evoked Ca^{2+} signals, and no combination of the three stimuli caused any greater inhibition than a single stimulus (Figure 4A,B). This suggests that the three stimuli converge to cause inhibition via a common pathway. Subsequent experiments resolve which of the two receptors (EP$_2$ or EP$_4$) mediates the response to the endogenous stimulus, PGE$_2$.

Inhibition of histamine-evoked Ca^{2+} signals by PGE$_2$ (pIC$_{50}$ = 9.27 ± 0.12, n = 4) was antagonized by AH6809 (30 μM, ΔpIC$_{50}$ = 0.62 ± 0.13), but to a lesser extent than the response to butaprost (ΔpIC$_{50}$ = 1.12 ± 0.15). GW627368X (1 μM) also inhibited the responses to PGE$_2$ (ΔpIC$_{50}$ = 0.24 ± 0.08), but to a much lesser degree than the response to L902,688 (ΔpIC$_{50}$ = 2.20 ± 0.28) (Figure 4C, Table 1). These results suggest that both EP$_2$ and EP$_4$ receptors contribute to the PGE$_2$-mediated inhibition of histamine-evoked Ca^{2+}

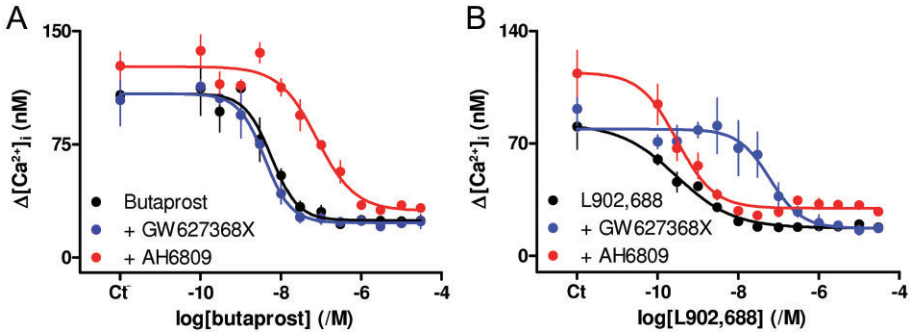

Figure 3

Activation of EP_2 or EP_4 receptors inhibits histamine-evoked Ca^{2+} signals. (A, B) Effect of butaprost (A, 5 min) or L902,688 (B, 5 min) on the Ca^{2+} signals evoked by histamine (3 μM) alone or in the presence of AH6809 (30 μM, 5 min) or GW627368X (1 μM, 5 min). Results (means ± SEM from three independent plates, with two wells analysed from each) show amplitudes of the peak changes in $[Ca^{2+}]_i$ evoked by histamine. Ct denotes control. Summary results are shown in Table 1.

Table 1

Effects of PGE_2 and selective ligands of EP_2 and EP_4 receptors on histamine-evoked Ca^{2+} signals and cAMP accumulation

	$[Ca^{2+}]_i$, pIC_{50} (/M)			cAMP, pEC_{50} (/M)
	Control	AH6809	GW627368X	Control
PGE_2	9.27 ± 0.12 (4)	8.65 ± 0.07* (4)	9.03 ± 0.13 (4)	6.62 ± 0.10 (4)
Butaprost	8.22 ± 0.05 (3)	7.10 ± 0.19* (3)	8.43 ± 0.17 (3)	5.68 ± 0.09 (4)
L902,688	9.52 ± 0.25 (3)	9.53 ± 0.05 (3)	7.31 ± 0.21* (3)	7.76 ± 0.11 (3)

Concentration-dependent effects of the indicated agonists of EP receptors on the increase in $[Ca^{2+}]_i$ evoked by histamine (3 μM) (pIC_{50}) or cAMP accumulation (pEC_{50}) are shown alone or in the presence of antagonists of EP_2 (AH6809, 30 μM) or EP_4 (GW627368X, 1 μM) receptors. Measurements of cAMP accumulation were performed using the HitHunter HS+ assay. Results are means ± SEM, with the number of independent experiments shown in parentheses.
*$P < 0.05$ relative to control.

signals, but the magnitudes of the disparities between the effects of the competitive antagonists on responses to PGE_2 and those evoked by the subtype-selective agonists (Figure 4D) suggest that EP_2 receptors mediate most responses to PGE_2. EP_4 receptors bind PGE_2 with approximately sixfold greater affinity than EP_2 receptors (Abramovitz *et al.*, 2000). The predominant role of the latter must, therefore, be due to their greater expression or more effective coupling to downstream signalling, consistent with our suggestion that only EP_2 receptors have a receptor reserve. Our evidence that transcripts for EP_2 and EP_4 receptors are expressed at similar levels would be consistent with more effective coupling of EP_2 receptors.

EP_2 and EP_4 receptors differ in their abilities to stimulate AC

Because EP_2 and EP_4 receptors share an ability to activate AC via the G protein Gs (Alexander *et al.*, 2011), we examined the effects of their activation on cAMP accumulation in human ASMC. PGE_2 evoked a concentration-dependent accumulation of cAMP whether assessed using ^{3}H-adenine labelling (pEC_{50} = 6.67 ± 0.06, n = 3) or the HitHunter assay (pEC_{50} = 6.62 ± 0.10, n = 4) (Figure 5). This stimulation of cAMP accumulation was reduced by 83 ± 1% (n = 3) in the presence of inhibitors of AC, SQ 22536 (1 mM) and DDA (200 μM) (hereafter described as SQ/DDA) (Figure 5B). We note that as with histamine-evoked Ca^{2+} signals, there was a variability between patients and cell passages in the absolute amounts of cAMP produced in response to PGE_2, again dictating the need for paired comparisons of treatments (Table 1). These results are consistent with an earlier report in which PGE_2, but not histamine, evoked an increase in cAMP in human ASMC (Satoh *et al.*, 1994). Butaprost also stimulated AC activity (pEC_{50} = 5.68 ± 0.09, n = 4; Figure 5A, Table 1). The similar maximal effects of PGE_2 and butaprost, and the ~10-fold higher EC_{50} of the latter, are consistent with evidence that butaprost is a full agonist of human EP_2 receptors with ~20-fold lower affinity than PGE_2 (Narumiya *et al.*, 1999; Abramovitz *et al.*, 2000) (Supporting Information Table S4).

L902,688, which has approximately twofold greater affinity than PGE_2 for EP_4 receptors (Supporting Information Table S4), stimulated a concentration-dependent (pEC_{50} = 7.76 ± 0.11) accumulation of cAMP that was only 32 ± 2% (n = 3) of that evoked by a maximally effective concentration of PGE_2 (Figure 5A). The lesser maximal response to L902,688

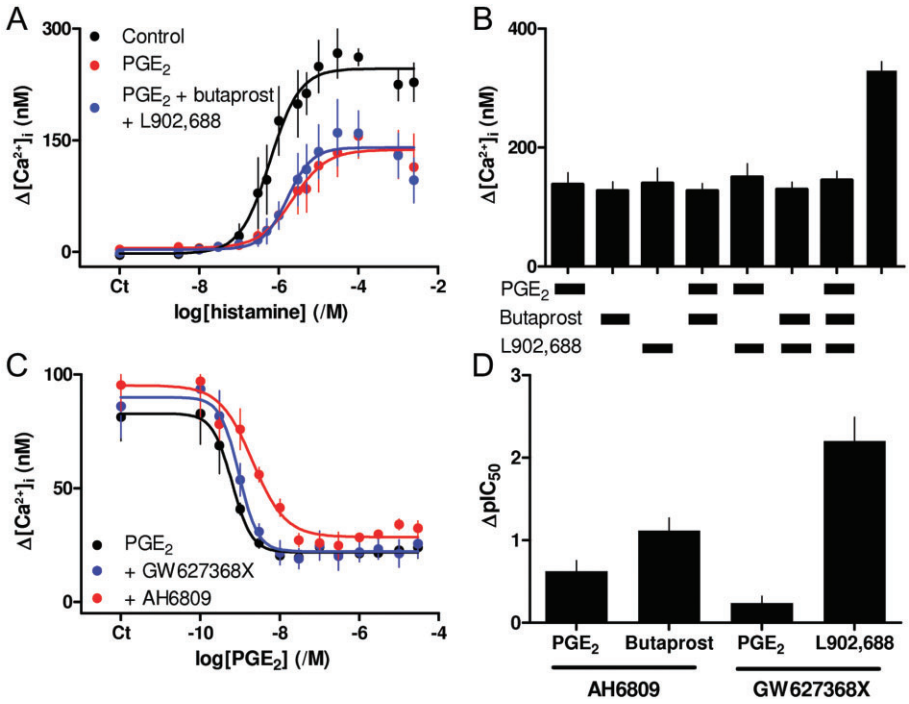

Figure 4

PGE_2 inhibits histamine-evoked Ca^{2+} signals largely via EP_2 receptors. (A) Effect of PGE_2 (10 μM) alone or in combination with butaprost and L902,688 (10 μM each) on the peak Ca^{2+} signals evoked by the indicated concentrations of histamine. Results are means ± SEM from three independent plates, each with one to two wells. (B) Peak Ca^{2+} signals evoked by histamine (3 μM) alone or with (5 min) PGE_2 (10 μM), butaprost (100 μM), L902,688 (10 μM) or the indicated combinations. Results are means ± SEM from 11 to 14 wells. (C) Effect of PGE_2 (5 min) on the peak Ca^{2+} signals evoked by histamine (3 μM) alone or in the presence of AH6809 (30 μM, 5 min) or GW627368X (1 μM, 5 min). Results are means ± SEM from four independent plates, each with one to two wells. (A, C) Ct denotes control. (D) ΔpIC_{50} values for PGE_2, butaprost and L902,688 and the antagonists of EP_2 (AH6809, 30 μM) or EP_4 (GW627368X, 1 μM) receptors. Results are means ± SEM from three to four independent experiments.

and greater sensitivity relative to PGE_2 (Figure 5A) suggest that EP_4 receptors probably mediate the effect of L902,688 on AC activity (Supporting Information Table S4). We suggest that both EP_2 and EP_4 receptors stimulate AC in human ASMC, but the former is either more abundant or more effectively coupled to AC, and largely mediates the effects of PGE_2. More effective coupling of EP_2 receptors would be consistent with our analysis of transcripts for EP_2 and EP_4 receptors, and with analyses showing that EP_2 receptors evoke greater stimulation of AC than do EP_4 receptors when each is heterologously expressed at a similar level (Fujino *et al.*, 2002).

Discussion

Activation of histamine H_1 receptors evokes an increase in $[Ca^{2+}]_i$ in human ASMC that results from Ca^{2+} release via IP_3 receptors, followed by a small sustained response mediated by Ca^{2+} entry, most likely via a store-operated Ca^{2+} entry pathway (Figure 1). Such biphasic Ca^{2+} signals are typical of those evoked by PLC-coupled receptors in VSM (Berridge, 2008) and other tissues (Putney, 1997). PGE_2 is another inflammatory mediator with widespread actions in the vasculature (Norel, 2007). The receptors through which PGE_2 regulates human ASMC have not hitherto been defined, although both EP_2 and EP_4 receptors mediate relaxation and/or attenuation of Ca^{2+} signalling in other human smooth muscles (Baxter *et al.*, 1995; Jones *et al.*, 1997; Benyahia *et al.*, 2012).

We have shown that in human ASMC, PGE_2 attenuates histamine-evoked Ca^{2+} signals without affecting the Ca^{2+} content of the intracellular stores (Figure 2). This inhibition is mediated by cAMP (Pantazaka *et al.*, unpubl. obs.). Inhibition of histamine-evoked Ca^{2+} signals is mimicked by selective activation of either EP_2 or EP_4 receptors (Figures 3 and 4A–C), but results with selective antagonists suggest that EP_2 receptors predominantly mediate the effect of PGE_2 on $[Ca^{2+}]_i$ (Figure 4D). This is consistent with the relationships between pIC_{50} and published K_D values for the subtype-selective agonists suggesting a greater receptor reserve for EP_2 receptors. It also aligns with evidence that both EP_2 and EP_4 receptors stimulate AC activity, but the maximal response is much larger for EP_2 receptors (Figure 5A). The predominant role of EP_2, relative to EP_4, receptors, despite the lower K_D of the latter (Supporting Information Table S4), could arise from higher levels of expression of EP_2 receptors in human ASMC or their more effective coupling to stimulation of AC (Fujino *et al.*, 2002). The latter seems more likely because transcripts for EP_2 and EP_4 receptors are expressed at similar levels in human ASMC. There is some evidence that functional EP

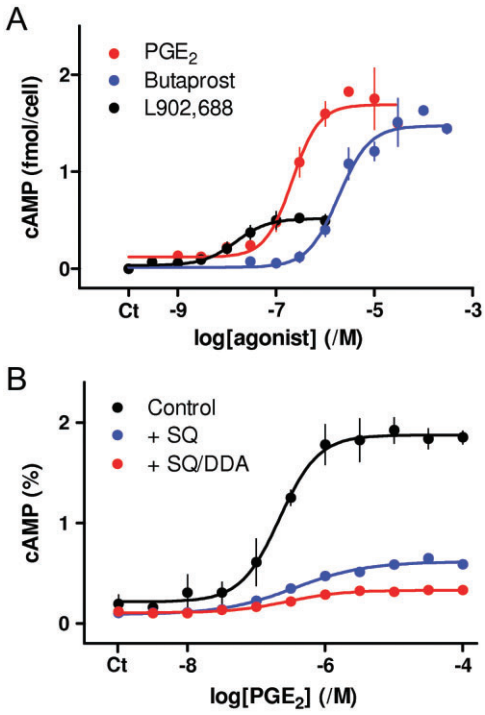

Figure 5

PGE_2 stimulates AC predominantly via EP_2 receptors. (A) Effects of the indicated concentrations of PGE_2, butaprost and L902,688 (5 min) on cAMP formation measured using a HitHunter assay. (B) Effect of PGE_2 (5 min) on cAMP accumulation (determined using ^{3}H-adenine labelling, and expressed as percentages of ^{3}H-ATP + ^{3}H-ADP) alone or after treatment with SQ 22536 (1 mM SQ) alone ($n = 1$) or SQ 22536 with DDA (200 μM) ($n = 3$). Both inhibitors were added 20 min before and then during stimulation with PGE_2. Results (except for SQ alone in panel B) are means ± SEM from three to four independent plates. Ct denotes control.

receptors, including EP_2 and EP_4 receptors, are expressed in both the plasma membrane and the nuclear envelope of some cells (Zhu *et al.*, 2006). We have not assessed whether this contributes to the ability of PGE_2 to inhibit histamine-evoked Ca^{2+} signals.

Our results demonstrate that two important inflammatory mediators, histamine and PGE_2, acting via receptors that are targets of existing clinically approved drugs, interact to control $[Ca^{2+}]_i$ in human ASMC. EP_2, EP_4 and histamine H_1 receptors might thereby provide effective therapeutic targets for treatment of atherosclerosis.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Inhibition of histamine-evoked Ca^{2+} signals by PGE_2 in ASMC from different patients. (A–C) Peak Ca^{2+} signals evoked by the indicated concentrations of histamine alone or with PGE_2 (10 μM added 5 min before histamine). Results are from three different patients (codes in Supporting Information Table S1) and show means ± SEM from five (A), 15 (B) and 11 (C) independent experiments. (D). Effect of the indicated concentrations of PGE_2 (added 5 min before histamine) on the pEC_{50} for histamine-evoked peak Ca^{2+} signals. Ct denotes control.

Figure S2 Inhibition of histamine-evoked Ca^{2+} signals and depletion of intracellular Ca^{2+} stores by 2-APB. (A, C) Effect of 2-APB (added 5 min before histamine) on the Ca^{2+} signals evoked by submaximal (3 μM) (A) or maximal (1 mM) (C) concentrations of histamine in Ca^{2+}-free HBS. (E) Similar analyses of the effects of 2-APB on the Ca^{2+} content of the intracellular stores assessed by addition of ionomycin (1 μM). The code shown in panel E applies to all three panels (B, D, F). Summary results show peak Ca^{2+} signals evoked by histamine (B, D) or ionomycin (F) after the indicated treatments with 2-APB. Results (A–F) are means ± SEM from three independent plates, with two wells analysed from each.

Figure S3 Caffeine and PGE_2 do not directly affect $[Ca^{2+}]_i$, and neither EP_3, DP_1 nor FP receptors contribute to inhibition of Ca^{2+} signals by PGE_2. (A) Populations of ASMC in HBS were stimulated with caffeine (A, 10 mM) and then histamine (3 μM). (B) In similar experiments, ASMCs were stimulated with histamine alone (3 μM) or after pretreatment with PGE_2 (10 μM) as shown. The trace shows that although PGE_2 inhibits histamine-evoked Ca^{2+} signals, it does not itself affect $[Ca^{2+}]_i$. Results (A and B) are means ± SEM from four wells on a single 96-well plate and are typical of results from three independent experiments. (C) The peak increase in $[Ca^{2+}]_i$ evoked by histamine (3 μM) is shown for histamine alone or in the presence of PGE_2 (100 nM) or agonists selective for EP_3 receptors (sulprostone, 1 μM), DP_1 receptors (BW 245C, 10 nM) or FP receptors (latanoprost, 100 nM) (each added 5 min before histamine). Results are means ± SEM from 8–29 wells.

Table S1 Sources of the human ASMC used.

Table S2 Key properties of the drugs used.

Table S3 Attenuation of histamine-evoked Ca^{2+} signals in human ASMC from different patients.

Table S4 Reported affinities (pK_D) of drugs used for analysis of EP_2 and EP_4 receptors.